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The Envelope Glycoprotein of Human Immunodeficiency Virus Type 2 Enhances Viral Particle Release: a Vpu-Like Factor?

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The Vpu protein is a human immunodeficiency virus type 1 (HIV-1)-specific accessory protein that is required for the efficient release of viral particles from infected cells. Even though HIV-2 does not encode Vpu, we found that this virus is nevertheless capable of efficiently releasing virus particles. In fact, the rate of virus release from HeLa cells transfected with a full-length molecular clone of HIV-2, ROD10, was comparable to that observed for the *vpu*$^+$ HIV-1 NL4-3 isolate and was not further enhanced by expression of Vpu in *trans*. However, consistent with previous observations showing that HIV-2 particle release is Vpu responsive in the context of HIV-1/HIV-2 chimeric constructs, exchanging the *gag-pol* region of NL4-3 with the corresponding region from pROD10 rendered the resulting chimeric virus Vpu responsive. Our finding that the responsiveness of HIV-2 particle release to Vpu is context dependent suggested the presence of a Vpu-like factor(s) encoded by HIV-2. Using chimeric proviruses encoding HIV-2 *gag* and *pol* in the context of the HIV-1 provirus that were coexpressed with subgenomic HIV-2 constructs, we found that the HIV-2 envelope glycoprotein had the ability to enhance HIV-2 particle release with an efficiency comparable to that of the HIV-1 Vpu protein. Conversely, inactivation of the HIV-2 *env* gene in the original ROD10 clone resulted in a decrease in the rate of viral particle release to a level that was comparable to that of Vpu-deficient HIV-1 isolates. Providing the wild-type envelope in *trans* rescued the particle release defect of the ROD10 envelope mutant. Thus, unlike HIV-1, which encodes two separate proteins to regulate virus release or to mediate viral entry, the HIV-2 Env protein has evolved to perform both functions.

Among all known primate and nonprimate lentiviruses, only human immunodeficiency virus type 1 (HIV-1) encodes the Vpu accessory protein (10, 32, 50), except for the chimpanzee simian immunodeficiency virus isolate SIVcpz, which contains a gene similar to *vpu* (21). Vpu is an 81-residue integral membrane protein that is posttranslationally modified by phosphorylation and has the ability to form oligomeric structures in membranes (13, 30, 45, 46, 49). Although not essential for virus replication in vitro, the expression of Vpu has been shown to enhance viral particle release from infected cells in tissue culture systems (49–51). In the absence of Vpu, viral proteins accumulate in infected cells as a result of intracellular budding, a phenomenon that is accompanied by increased cytopathicity of *vpu*-deficient isolates (25). More recent studies provide evidence that the effect of Vpu on virus particle release may be correlated with an ion channel activity of its transmembrane domain (43). Vpu has also been shown to induce specifically the degradation of CD4 trapped in the endoplasmic reticulum (ER) of cells by the envelope glycoprotein precursor gp160 (27, 52, 55, 56). Even though Vpu and Env are coexpressed from a bicistronic mRNA (48), the gp160 envelope product does not appear to be directly involved in either biological activities of Vpu (7, 14, 55, 57). Although the two biological functions of Vpu appear to converge and result in the increased production of infectious virions, they are distinct and mechanistically separable (for a review, see reference 4). The degradative activity of Vpu appears to be specific for CD4 (54), to involve physical interactions with target molecules (5), and to require Vpu phosphorylation at two conserved serine residues (12, 47). On the other hand, Vpu enhancement of particle release operates through a more generalized mechanism. Indeed, Vpu has been shown to augment release of chimeric viruses bearing the *gag-pol* regions of retroviruses that naturally lack a *vpu* open reading frame (ORF), such as HIV-2, visna virus, and Moloney murine leukemia virus (16).

In this report, we examine the efficiency of particle release and Vpu responsiveness of Gag-Pol proteins produced by a full-length molecular clone of HIV-2_{ROD} (pROD10). We show that in contrast to HIV-1/HIV-2 chimeric viruses, virus release from HeLa cells transfected with the ROD10 isolate is very efficient and cannot be enhanced by coexpression of Vpu in *trans*. However, in agreement with previous work (16), we found that in the context of an HIV-1 chimeric virus, the efficiency of HIV-2 particle release was reduced nearly threefold compared with wild-type ROD10 and became Vpu responsive. These results not only demonstrate that the Vpu responsiveness of HIV-2 Gag-Pol is context dependent but also suggest the presence of a Vpu-like factor(s) in HIV-2. Coexpression of subgenomic HIV-2 constructs with the HIV-2/HIV-1 chimera demonstrated that the HIV-2 *env* gene product has the ability to enhance viral particle release in a manner similar to that of Vpu. Indeed, mutations that disrupt the pROD10 *env* ORF, but not the *vif*, *vpr*, *vpx*, or *nef* ORF, have a profound negative effect on virus particle release. Concomitantly, the pROD10 envelope glycoprotein provided in *trans* can rescue the envelope mutants and restore wild-type levels of particle release. Taken together, our results suggest that in addition to its known role in CD4-dependent infection of target cells, the HIV-2 envelope glycoprotein has the ability to positively regulate virus particle release.

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MATERIALS AND METHODS

Recombinant plasmid DNA. Plasmid pROD10 is a full-length infectious molecular clone derived from the HIV-2_{ROD} isolate (8). The construction and characterization of pROD10 are described elsewhere (42).

The pNLgp2 chimeric proviral construct was obtained by replacing the *gag-pol* region of the HIV-1 molecular clone pNL4-3 (1) by the corresponding pROD10 sequences. The construction involved a multistep cloning strategy which ultimately introduced the pROD10 *Nar*I-*Xba*I fragment (positions 859 in the 5′ long terminal repeat [LTR] and 5528 in *vif*, respectively) into pNL4-3 between the common *Nar*I site at position 637 in the 5′ leader region and a PCR-generated *Xba*I site at position 4830 upstream of the *vif* ORF. Base numbering for both pNL4-3 and pROD10 starts at the first nucleotide in the 5′ LTR. Both the exon 2 splice acceptor and donor sites at the end of the HIV-1 *pol* gene (37) were maintained in pNLgp2, thus allowing the synthesis of all downstream HIV-1 proteins, including Vif. The Vpu$^-$ counterpart of pNLgp2 (pNLgp2/Udel-1) was constructed by transferring a 1,424-bp *Sal*I-*Nhe*I fragment from plasmid pNL4-3/Udel-1 (originally termed vpuDEL-1 [25]) into pNLgp2.

Plasmid pNL-A1 is a derivative of pNL4-3 that lacks the *gag* and *pol* genes but expresses all other HIV-1 genes (50). The *vpu*-deficient plasmid pNL-A1/Udel-1 was constructed by introducing a 562-bp *Eco*RI-*Kpn*I fragment from pNL4-3/Udel-1 into pNL-A1.

pROD-A1 is an HIV-2_{ROD} equivalent of pNL-A1 that expresses all HIV-2 proteins except Gag and Pol and was derived from pROD10. The *gag-pol* deletion was performed in several steps that resulted in the removal of sequences between the *Bgl*I (between the major splice donor and the *gag* initiation codon) and *Bcl*I (positions 1051 to 5220) sites, leaving 270 nucleotides of untranslated *pol* sequences that partially overlap the *vif* gene.

pCM5-Env is a Rev-dependent construct that expresses the full-length ROD10 envelope protein under the transcriptional control of the cytomegalovirus early promoter. To generate the pCM5-Env construct, a 2,226-bp fragment consisting of the full-length *env* gene flanked by an *Xba*I site at the 5′ end and a *Bam*HI site at the 3′ end was amplified by PCR. The PCR product was digested with *Xba*I-*Bam*HI and cloned under the control of the cytomegalovirus promoter by using the *Xba*I and *Bgl*II sites of the previously described plasmid pCMV-CD8/cyto4 (54). This strategy removed all CD8 and CD4 sequences that were replaced with the ROD10 *env* gene.

The pROD-A1.env1 plasmid is a frameshift mutant that produces a truncated protein consisting of only the first 60 N-terminal Env amino acids plus 15 missense residues and was created by inserting a 10-bp *Xho*I linker (New England Biolabs, Beverly, Mass.) into the *Bsa*BI site at position 2710 in pROD-A1. The envelope mutation from pROD-A1.env1 was transferred to the full-length molecular clone pROD10. pROD10.env1 was constructed by inserting a 3,359-bp *Sap*I-*Bsm*I fragment from pROD-A1.env1 into pROD10.

Mutations were introduced into the *vif*, *vpr*, *vpx*, and *nef* genes of pROD10. Each of the pROD10.vif, pROD10.vpr, and pROD10.nef mutants consisted of four-base insertions at the *Xba*I, *Hin*dIII, and *Nco*I restriction sites, respectively, in pROD10 (44). The *vpx1* mutation has been described earlier (19) and was introduced in pROD10 by transferring the *Hin*dIII fragment from pROD9 into pROD10. The presence of all mutations was confirmed by DNA sequence analysis.

Cells and transfection. HeLa cells (ATCC CCL2) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). For transfection, HeLa cells were grown to near confluence in 25-cm^2 flasks (5×10^6 cells per flask). Two hours prior to transfection, the medium was replaced with 5 ml of fresh DMEM-FBS. Calcium phosphate-precipitated (18) plasmid DNA (25 to 30 μg) was added to the cells. The medium was removed after 4 h, and the cells were subjected to a glycerol shock for 2.5 min as described previously (15). The cultures were then washed once with phosphate-buffered saline (PBS) and maintained in 5 ml of DMEM-FBS.

Antisera and antibodies. Serum from an asymptomatic HIV-1-seropositive patient (TP serum) was used to detect HIV-1-specific proteins, including Vpu, by immunoprecipitation. The TP serum also quantitatively recognizes the HIV-2_{ROD} mature core and matrix proteins but not the Gag precursor or the envelope proteins. A monoclonal antibody to HIV-2 (LAV-2) core protein (SmithKline Beecham Pharmaceuticals) and a rabbit antiserum to HIV-2_{ST} gp120 (Epitope, Inc.) were used for immunoprecipitation of HIV-2_{ROD} proteins not recognized by the TP serum. These reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases and were originally contributed by Paul Yoshihara and Raymond Sweet, respectively (20, 23, 34).

Pulse-chase and immunoprecipitation. Transfected HeLa cells were scraped off the flasks at 16 h posttransfection, washed once with PBS (10 mM phosphate buffer [pH 7.4], 100 mM NaCl), and starved for 15 min in methionine- and cysteine-free RPMI 1640 medium (Specialty Media, Inc., Lavallette, N.J.). Cells were pulse-labeled with 1 mCi of [^{35}S]methionine and [^{35}S]cysteine (Trans^{35}S-Label; ICN Biomedical, Inc., Costa Mesa, Calif.) per ml for 30 min. The medium was then removed, the cells were washed once in PBS, and equal aliquots (100 μl) were added to 200 μl of prewarmed DMEM-FBS for each time point of the chase period and incubated at 37°C. At the indicated time points, cells were collected, lysed in 400 μl of Nonidet P-40 (NP-40)–deoxycholate (DOC) buffer (20 mM Tris-HCl [pH 8], 120 mM NaCl, 2 mM EDTA, 0.5% DOC, 1% NP-40) and stored on dry ice. The culture supernatants collected at each time point were filtered through 0.45-μm-pore-size cellulose acetate Spin-X centrifuge tube filters (Corning Costar Corporation, Cambridge, Mass.) to remove cells and cell debris. Virus particles were then pelleted from cell-free supernatants in a refrigerated Eppendorf microcentrifuge (4°C, 90 min, 16,000 × *g*). Pelleted virions were lysed in 400 μl of NP-40–DOC buffer. Cell lysates were precleared by incubation at 4°C for 1 h with protein A-Sepharose beads (Sigma Chemical, St. Louis, Mo.). Immunoprecipitations were conducted as described previously (3, 55, 56). Immunoprecipitates were solubilized by boiling in sample buffer containing 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 1% glycerol, and 65 mM Tris-hydrochloride (pH 6.8) and separated on SDS–12% polyacrylamide gels. Gels were fixed for 30 min by incubation in 25% methanol–7% acetic acid, incubated for 20 min in Enlightning (NEN Research Products, Boston, Mass.), and dried. Radioactive bands were visualized by fluorography using Bio-Max MR films (Eastman Kodak, Rochester, N.Y.). Quantitation of the relevant bands was performed with a Fujix BAS 2000 Bio-Image Analyzer.

In experiments involving brefeldin A (BFA), cells were incubated for 2 h in DMEM-FBS supplemented with BFA (2.5 μg/ml). Cells were then washed once in methionine- and cysteine-free RPMI 1640 medium containing 2.5 μg of BFA per ml and incubated for 30 min in 1.5 ml of the same medium. Cells were then labeled for 30 min with Trans^{35}S-Label (1 mCi/ml; ICN) and chased for up to 6 h as described above. All steps were done in the presence of 2.5 μg of BFA per ml. During the chase period, fresh BFA (2 μg/ml) was added every 2 h.

RESULTS

Vpu responsiveness of HIV-2_{ROD} virus release is context dependent. HIV-1 Vpu was previously reported to affect virus particle release not only of HIV-1 but also of HIV-2, visna virus, and Moloney murine leukemia virus (16) even though these viruses do not encode a *vpu* gene. To confirm these observations, we analyzed the effect of Vpu on the release of particles expressed from a full-length molecular clone of HIV-2, pROD10. Pulse-chase experiments were performed in HeLa cells transfected with either pROD10 alone or in the presence of a twofold molar excess of the pNL-A1 plasmid DNA to provide wild-type Vpu in *trans* (47, 50). Cells were labeled and subjected to a chase for up to 6 h. At each time point, equal aliquots of cells were harvested and virions released into the supernatant were collected by centrifugation as described in Materials and Methods. Each fraction was lysed in lysis buffer, and viral proteins were subjected to immunoprecipitation with an HIV-1 patient serum containing antibodies against all major HIV-1 proteins supplemented with a monoclonal antibody against HIV-2 core proteins. Immunoprecipitated proteins were separated on SDS–12% polyacrylamide gels and visualized by fluorography (not shown). To estimate the efficiency of particle release, radioactive bands corresponding to the p58^{gag}, p26^{CA}, and p16^{MA} proteins were quantified, and the ratio of viral Gag proteins present in the culture supernatant to the total pool (cell and supernatant) was calculated and plotted as a function of time (Fig. 1A). Interestingly, despite the absence of Vpu, release of pROD10 particles was very efficient, exceeding 50% within the 6-h chase period. Moreover, providing Vpu in *trans* by cotransfection of plasmid pNL-A1 did not further enhance particle release from the pROD10 molecular clone, suggesting that particle release in this case is already maximal and therefore no longer responsive to Vpu. This result contrasts with previous observations that suggest that HIV-2 particle release is inefficient and is Vpu responsive (16).

Our experiments differ from those previously performed in that they involve the analysis of an infectious, full-length molecular clone of HIV-2 rather than HIV-1/HIV-2 chimeric viruses containing the HIV-2_{ROD} *gag-pol* region (16). To investigate whether the contrasting results are due to the different experimental designs, we performed a series of experiments involving HIV-1/HIV-2 chimeric proviruses. Plasmids encoding Vpu$^+$ and Vpu$^-$ chimeric viruses, designated pNLgp2 and pNLgp2/Udel-1, respectively, were constructed by replacing

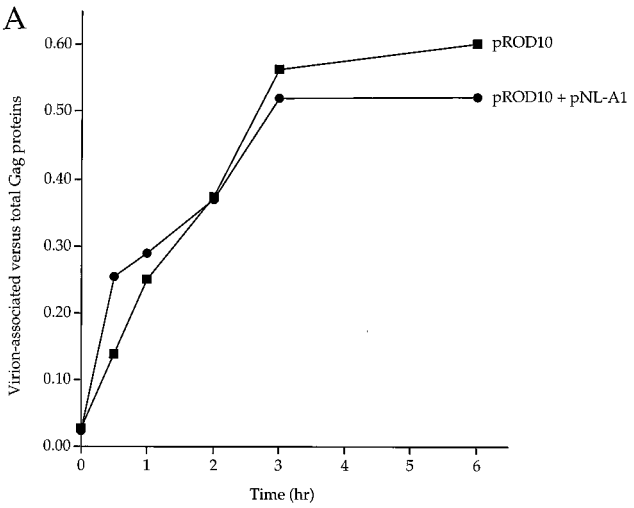
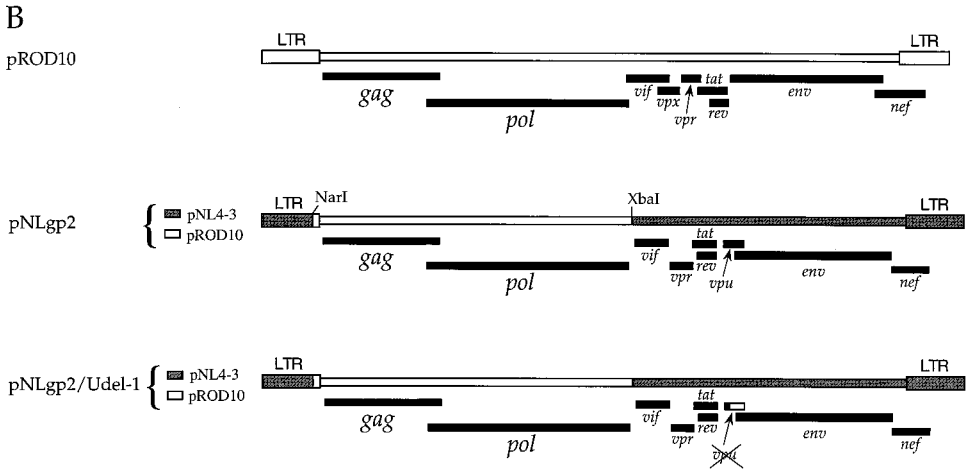
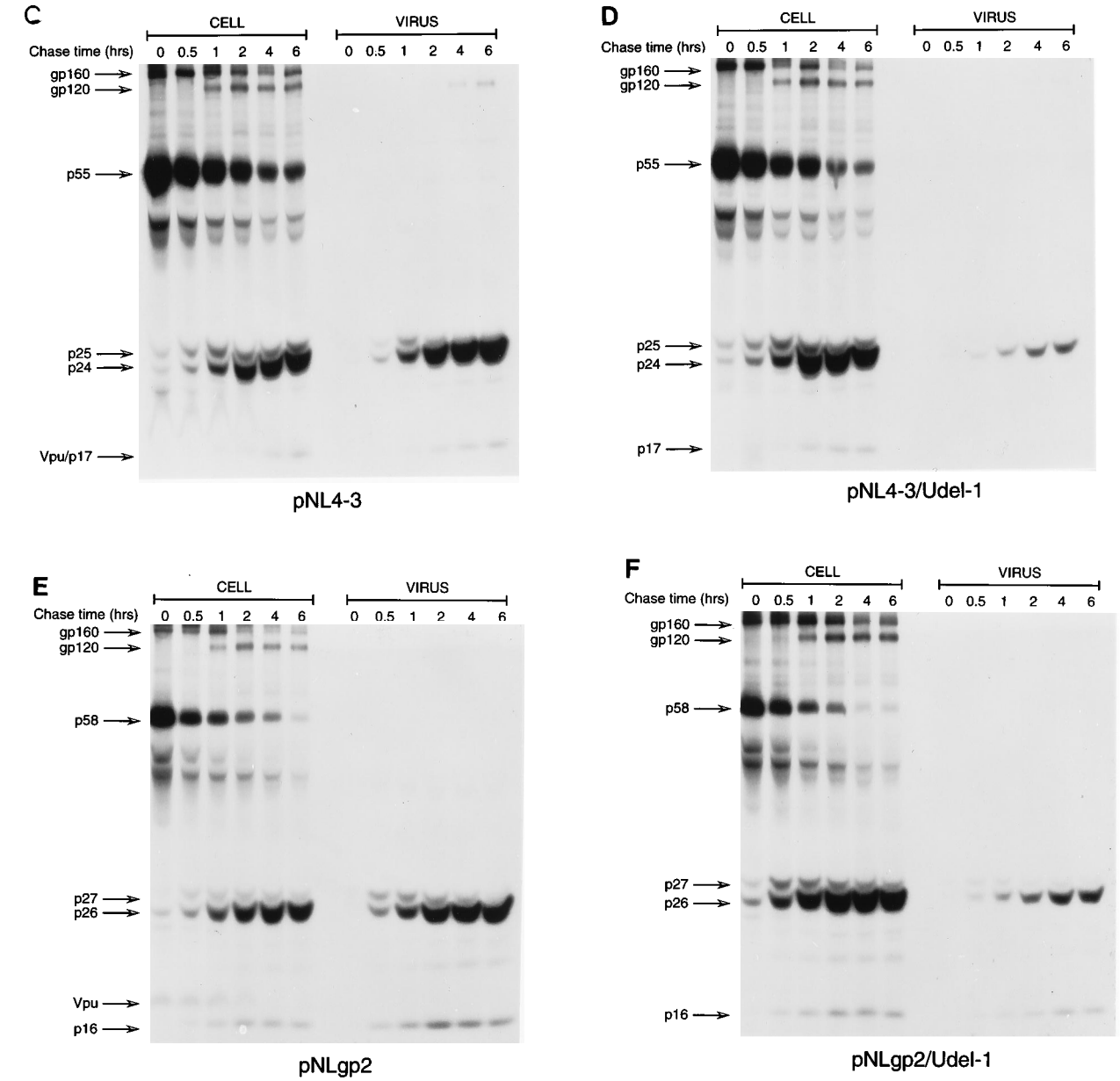


FIG. 1. Vpu responsiveness of HIV-2_{ROD} virus release is context dependent. (A) Kinetic analysis of viral particle release by pROD10 in the presence or absence of Vpu. HeLa cells were transfected with 15 μg of pROD10 DNA alone or together with 20 μg of pNL-A1 and subjected to pulse-chase analysis. Cells were pulse-labeled for 30 min with Trans^{35}S-Label (1 mCi/ml) and chased for the indicated times. At each time point, aliquots of the cells and the virus-containing supernatants were harvested, and virus particles present in the culture medium were pelleted. Cells and viral pellets were lysed in buffer containing NP-40 and DOC. Viral proteins were recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions, separated on SDS–12% polyacrylamide gels, and visualized by fluorography (not shown). Bands corresponding to the precursor and mature Gag proteins were quantified with an image analyzer. The efficiency of particle release at each time point was calculated by dividing the amount of Gag proteins present in the pelleted virus fraction by the total of cell- and virus-associated Gag proteins. The ratio of virion-associated to total Gag proteins was then plotted as a function of time. (B) Schematic representation of the pNLgp2 and pNLgp2/Udel-1 chimeras. Sequences from the pROD10 molecular clone of HIV-2 containing the *gag* and *pol* genes were introduced into pNL4-3, using the *Nar*I and *Xba*I sites shown. The pNLgp2/Udel-1 chimera is an isogeneic variant of pNLgp2 and carries a deletion that inactivates the *vpu* gene. (C to F) Kinetics of viral particle release by the pNLgp2 and pNLgp2/Udel-1 chimeric viruses. HeLa cells were transfected with 30 μg of plasmid DNA of pNL4-3 (C), pNL4-3/Udel-1 (D), pNLgp2 (E), and pNLgp2/Udel-1 (F). Pulse-chase analysis, immunoprecipitation, and gel electrophoresis were done as described above. Viral proteins detected on the gels are identified on the left. (G) Bands corresponding to HIV-1 p55^{gag}, p24-25^{CA}, and p17^{MA} in panels C and D or HIV-2 p58^{gag}, p26-27^{CA}, and p16^{MA} in panels E and F were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described above, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.



the NL4-3 *gag* and *pol* genes with the corresponding regions from pROD10 (Fig. 1B). HeLa cells were transfected with the Vpu-expressing pNLgp2 or the Vpu-defective pNLgp2/Udel-1 plasmid DNA, and the rate of particle release was assessed by pulse-chase analysis. Cells transfected with plasmid pNL4-3 or pNL4-3/Udel-1 encoding wild-type or Vpu-deficient HIV-1 were used as a positive or negative control, respectively. Cells were pulse-labeled and subjected to a chase for up to 6 h as described for Fig. 1A. Cell- and virus-associated viral proteins collected at various times were immunoprecipitated with an HIV-1-reactive patient serum supplemented with a serum containing antibodies against HIV-2 core proteins. Immunoprecipitated proteins were separated on SDS–12% polyacrylamide gels and visualized by fluorography (Fig. 1C to F). Radioactive bands corresponding to the HIV-1-specific p55^{gag}, p24-25^{CA}, and p17^{MA} proteins in Fig. 1C and D and to the HIV-2-specific p58^{gag}, p26-27^{CA}, and p16^{MA} proteins in Fig. 1E and F were quantified. The amounts of particle-associated proteins present at each time point were calculated relative to the total amounts of intra- and extracellular Gag proteins and were plotted as a function of time (Fig. 1G). In the case of pNL4-3, the Vpu effect on particle release was manifested by a nearly fivefold increase in the amount of virus secreted from cells over a 6-h period compared with the pNL4-3/Udel-1 clone (Fig. 1G). A similar Vpu effect was observed when virus release from cells transfected with pNLgp2 and pNLgp2/Udel-1 were compared, indicating that in the context of an HIV-1 provirus, HIV-2 particle release was Vpu responsive in a fashion similar to that of wild-type HIV-1 (Fig. 1G). The efficiency of particle release from both the pNLgp2 and pNLgp2/Udel-1 chimeras was somewhat higher than that of the wild-type counterparts. However, this does not account for the high particle release efficiency observed with pROD10 (Fig. 1A).

In cells transfected with pNL4-3 or the pNLgp2 chimera, expression of Vpu did not affect synthesis or processing of the Gag precursor but rather resulted in a redistribution of the Gag proteins from the intracellular to the extracellular pool (Compare Fig. 1C with 1D and Fig. 1E with 1F). Synthesis and processing of the HIV-1 envelope glycoprotein precursor gp160 from the chimeric constructs was also comparable to that of NL4-3 viruses and was unaffected by the presence or absence of Vpu (Fig. 1C to F). Identical results were obtained



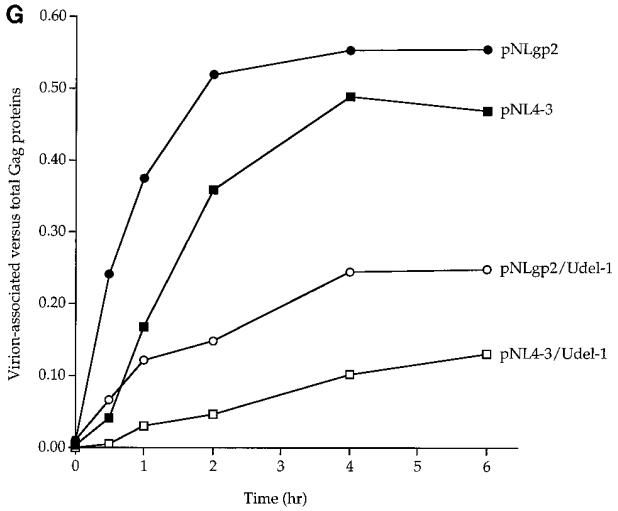


FIG. 1—*Continued.*

with chimeric viruses containing only the HIV-2_{ROD} *gag* and protease genes in the context of HIV-1, indicating that Vpu action on particle release is independent of the HIV-2 reverse transcriptase and integrase genes (data not shown). Taken together, these results confirm that in the context of HIV-1 chimeric viruses, release of HIV-2 particles is Vpu responsive (16). Since our chimeric constructs are based on a different HIV-1 isolate (NL4-3) than the HXBH10 chimeras used in previous studies (16), these results also suggest that the observed effect of Vpu on HIV-2 particle release is a general phenomenon and not restricted to a particular HIV-1 isolate.

HIV-2_{ROD} encodes a positive factor that enhances particle release in *trans*. The results of the experiments shown in Fig. 1 demonstrated that transfer of the HIV-2 *gag* and *pol* genes from their natural context into an HIV-1 provirus resulted in a significant decrease in the efficiency of virus production. It is thus likely that sequences outside the *gag-pol* region in pROD10 contribute to the efficient release of progeny virions. To investigate whether HIV-2 encodes a positive factor capa-

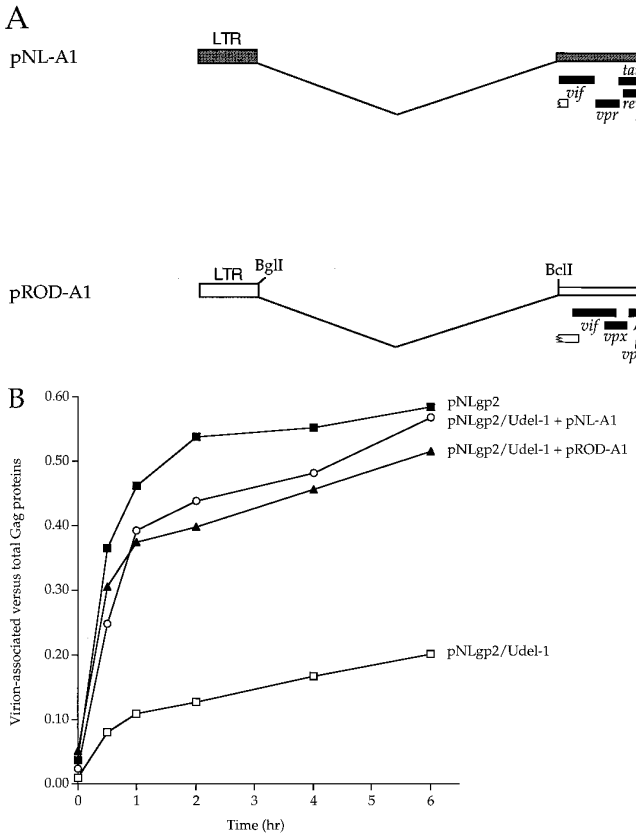


FIG. 2. HIV-2_{ROD} encodes a Vpu-like factor. (A) Schematic representation of the pNL-A1 (HIV-1) and pROD-A1 (HIV-2) subviral constructs. Plasmid pNL-A1 is a derivative of pNL4-3 that lacks the *gag* and *pol* genes but expresses all other HIV-1 genes (50). The pROD-A1 vector is structurally similar to pNL-A1 and was derived from the pROD10 molecular clone by deleting the *gag* and *pol* genes as described in Materials and Methods. The constructs are under the transcriptional control of the pNL4-3 and pROD10 LTRs, respectively. (B) Effect of pNL-A1 and pROD-A1 on the kinetics of viral particle release by pNLgp2/Udel-1-transfected cells. HeLa cells were transfected with 20 μg of pNLgp2/Udel-1 plasmid DNA in the presence of 15 μg of either pNL-A1 or pROD-A1. Cells transfected with 20 μg of pNLgp2 and pNLgp2/Udel-1 alone were included as positive and negative controls, respectively. Viral particle release was assessed by pulse-chase analysis as described for Fig. 1. Gag proteins recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.

ble of enhancing virus secretion, a subviral vector, pROD-A1, was constructed. The structure of this plasmid is analogous to that of the HIV-1 construct pNL-A1 and allows the expression of all HIV-2 genes except *gag* and *pol* (Fig. 2A). To analyze the effect of HIV-2 proteins on particle release from HIV-1/HIV-2 chimeras, HeLa cells were transfected with pNLgp2/Udel-1 alone or in the presence of either pROD-A1 or pNL-A1 plasmid DNA (1:1 molar ratio). As a control, virus production by pNLgp2, which expresses Vpu, was analyzed in parallel. Particle release was analyzed by pulse-chase analysis as described for Fig. 1. Cell- and virus-associated Gag proteins immunoprecipitated at each time point were separated on SDS–12% polyacrylamide gels (not shown), and the efficiency of particle release was calculated after quantification of the radioactive bands with an image analyzer (Fig. 2B). Consistent with the results above (Fig. 1G), particle release from the Vpu-deficient pNLgp2/Udel-1 chimera was inefficient relative to release from pNLgp2 but could be enhanced by providing Vpu in *trans* (Fig. 2B). Coexpression of pROD-A1 with pNLgp2/Udel-1 also resulted in an enhancement of particle release that was remarkably similar to the effect observed with pNL-A1 (Fig. 2B). Thus, despite the absence of any *vpu* sequences, pROD-A1 was able to enhance HIV-2 particle release in a manner indistinguishable from that by Vpu. Although Vpu functions in *cis* and in *trans*, complementation of pNLgp2/Udel-1 by pNL-A1 was slightly less efficient than expression of Vpu from pNLgp2. It is possible that the effect of Vpu on particle release, similar to that on CD4 degradation (27), is concentration dependent and that Vpu was expressed in limiting quantities from pNL-A1 in this experiment.

Taken together, these results provide strong evidence that HIV-2 encodes a Vpu-like factor that facilitates virus secretion when provided in *trans*. This finding may also explain the difference between the efficiency of HIV-2 particle release in the context of HIV-1 chimeras and that in the full-length pROD10 molecular clone since the chimeric constructs lack the activating element(s) encoded by pROD10 and pROD-A1.

The HIV-2_{ROD} Vpu-like factor is sensitive to BFA treatment. Vpu is an integral membrane protein whose effect on virus release can be blocked by treatment of cells with BFA (47). Since BFA treatment of cells results in the retention of Vpu in the ER (55), sensitivity of virus secretion to BFA implies that this process requires the presence of Vpu in a post-ER compartment (47). We therefore addressed whether the Vpu-like activity observed in pROD-A1 is also catalyzed by a membrane-associated factor that might be sensitive to BFA treatment. To test this hypothesis, HeLa cells transfected with pROD10 were pulse-labeled, subjected to a 6-h chase, and analyzed as described for Fig. 1 except that BFA was added to one set of the samples. Radioactive bands corresponding to cell- and virus-associated Gag proteins were quantified, and the efficiency of particle release was calculated as described for Fig. 1 (Fig. 3). BFA decreased the efficiency of pROD10 particle release nearly threefold over the 6-h period. From the seven ORFs encoded by pROD-A1, only the gp140 envelope glycoprotein is known to be an integral membrane protein synthesized in the ER (39), and its transport through the ER-Golgi compartments is presumably sensitive to BFA treatment. In light of this result, the envelope protein is thus the most likely candidate for the Vpu-like function we identified in pROD10 and pROD-A1.

Inactivation of the HIV-2_{ROD} envelope gene but not the *vif*, *vpr*, *vpx*, or *nef* gene decreases the efficiency of particle release. To assess directly the potential activity of the HIV-2 Env protein on particle release, we first examined whether an envelope mutant of pROD-A1, pROD-A1.env1, was still able to en-

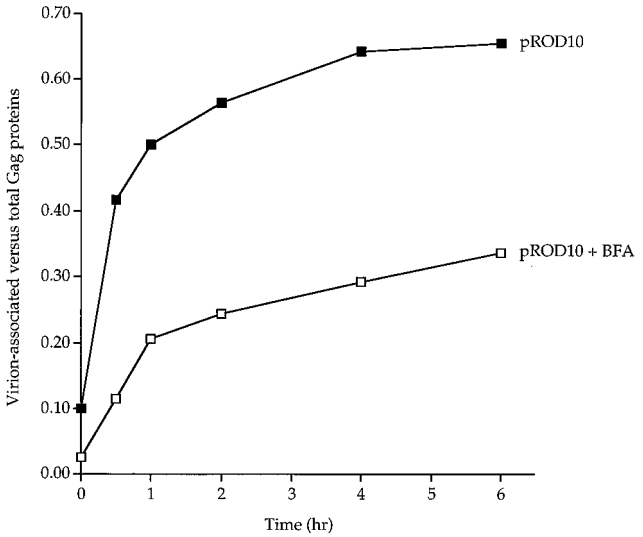


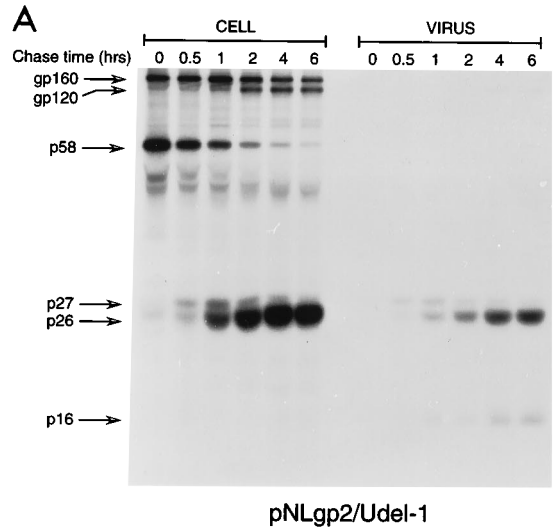
FIG. 3. BFA inhibits secretion of HIV-2_{ROD} particles. HeLa cells were transfected with 20 μg of pROD10 plasmid DNA and subjected to pulse-chase analysis as described for Fig. 1 (pROD10). Half of the cells from the same transfection were pretreated with 2.5 μg of BFA per ml for 2 h prior to pulse-labeling and maintained in 2 μg of BFA per ml for the duration of the chase (pROD10 + BFA). Viral Gag proteins recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described for Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.

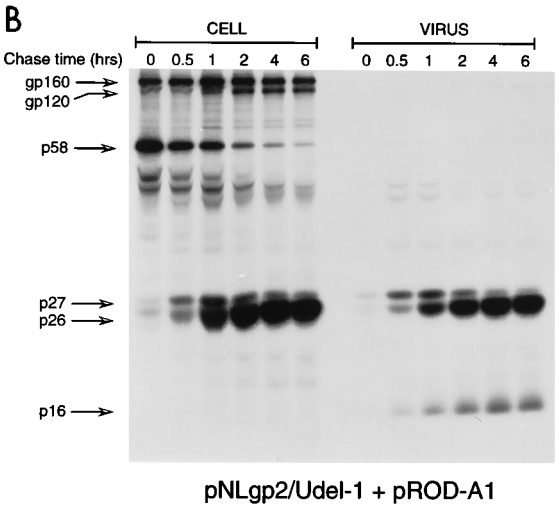
hance particle release directed by pNLgp2/Udel-1. The pROD-A1.env1 plasmid is a variant of pROD-A1 that produces a truncated envelope glycoprotein of the N-terminal 60 residues. HeLa cells were transfected with pNLgp2/Udel-1 either alone or together with either pROD-A1 or pROD-A1.env1. Transfected cells were pulse-labeled and chased for up to 6 h as described above. Cell- and virus-associated viral proteins collected at different times were immunoprecipitated with a mixture of HIV-1 and HIV-2 sera, separated on SDS–12% polyacrylamide gels, and visualized by fluorography (Fig. 4A to C). Radioactive bands corresponding to Gag proteins in Fig. 4A to C were quantified, and the effects of pROD-A1 and pROD-A1.env1 on the efficiency of pNLgp2/Udel-1 particle release were calculated as described for Fig. 1 (Fig. 4D). Consistent with results of Fig. 2B, pROD-A1 was able to enhance particle release directed by the pNLgp2/Udel-1 chimera (Fig. 4D). In contrast, the pROD-A1.env1 plasmid, bearing a stop codon in the envelope gene, lost the ability to enhance pNLgp2/Udel-1 particle release (Fig. 4D). The inability of pROD-A1.env1 to enhance pNLgp2/Udel-1-directed virus secretion suggests that the envelope gene of pROD-A1 has an important function for the efficient secretion of HIV-2 particles.

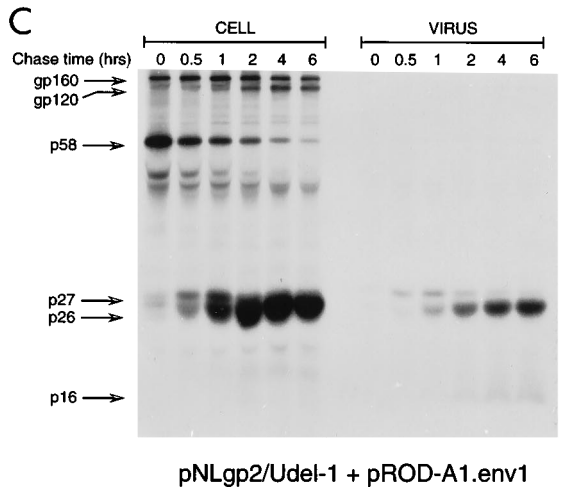
To confirm the effect of the HIV-2 Env protein on virus production in the context of the full-length molecular clone pROD10, the envelope mutation present in pROD-A1.env1 was transferred to pROD10, and the efficiency of virus production directed by the resulting pROD10.env1 mutant was compared with that of pROD10. Pulse-chase experiments were performed as described above in HeLa cells transfected with either pROD10 or the pROD10.env1 mutant (Fig. 4E and F). Mutation of the HIV-2 *env* gene, apparent by the absence of the gp140 envelope glycoprotein in cells transfected with the mutant pROD10.env1, had no effect on the synthesis and processing kinetics of the HIV-2_{ROD} Gag proteins compared with wild-type pROD10 (Fig. 4E and F, cell). However, mutation of the envelope protein had a significant negative effect on pROD10 virus secretion, consistent with results obtained with the pROD-A1.env1 mutant (Fig. 4E and F, virus). Taken together, these results indicate that the envelope glycoprotein of HIV-2_{ROD} has the ability to enhance viral particle release in a manner similar to that of Vpu, both in the context of HIV-1 chimeric viruses and in the full-length pROD10 molecular clone. To rule out the potential contribution of other gene products encoded by pROD10 for virus release that might act in conjunction with the envelope glycoprotein, we tested the effects of mutations in the *vpx*, *vpr*, *vif*, and *nef* ORFs on particle release directed by pROD10. Isogenic variants of pROD10 deficient in any one of these ORFs were constructed, and the rate of particle release directed by these mutants was compared with that directed by pROD10 and pROD10.env1 in pulse-chase experiments as described above. None of the mutations was found to alter synthesis and maturation of viral proteins (data not shown). In addition, quantitation of the gels demonstrates that particle release directed by pROD10 is unaffected by mutations in the *vpx*, *vpr*, *vif*, and *nef* ORFs (Fig. 4G). Only truncation of the env gene caused a decrease in the efficiency of ROD10 viral particle release (Fig. 4G). The gp140 envelope glycoprotein of HIV-2_{ROD} thus appears to have an essential function in the process of HIV-2_{ROD} viral particle formation and release.

To further confirm that the decreased efficiency of particle release observed for the pROD10.env1 mutant in Fig. 4G was due to inactivation of the envelope gene, we addressed whether normal levels of particle release could be restored by providing the envelope protein in *trans*. HeLa cells were transfected with the pROD10.env1 plasmid in the presence of either pROD-A1 or pCM5-Env, the latter construct expressing wild-type ROD10 Env protein under the transcriptional control of the cytomegalovirus promoter in the absence of any other HIV-2 gene product. Cells cotransfected with pROD10.env1 and pROD-A1.env1 were used as a negative control in this experiment. Pulse-chase experiments were performed as described in Materials and Methods except that the cells were subjected to a 4-h chase. Cell- and virus-associated viral proteins collected at 0, 1, 2, and 4 h postlabeling were immunoprecipitated with a mixture of HIV-1 and HIV-2 sera, separated on SDS–12% polyacrylamide gels, and visualized by fluorography (data not shown). Radioactive bands corresponding to Gag proteins were quantified, and the effect of pROD-A1, pROD-A1.env1, or pCM5-Env on pROD10.env1 particle release was calculated as described above. As shown in Fig. 4H, plasmid pROD-A1 could restore the particle release defect exhibited by the pROD10.env1 envelope mutant. Cotransfection of pROD10.env1 and the Env expression plasmid pCM5-Env led to a similar increase of virus release (Fig. 4H). In contrast, cotransfection of the envelope-deficient plasmid pROD-A1.env1 did not enhance the efficiency of pROD10.env1 particle release. We therefore conclude that the envelope protein of HIV-2_{ROD10} is necessary and sufficient to restore the particle release defect exhibited by the pROD10.env1 mutant.

Taken together, results presented in Fig. 4 show that the envelope glycoprotein of HIV-2_{ROD10} enhances the release of HIV-2 particles both in the context of an HIV-1 chimera and in the full-length ROD10 isolate. Although we do not exclude at present that this Vpu-like activity requires the presence of cellular factors, we have ruled out the contribution of each of the *pol*, *vif*, *vpx*, *vpr*, and *nef* genes in this process. Interestingly, particle release by the ROD10.env1 mutant was very similar to release by ROD10 following BFA treatment (Fig. 3) and pNLgp2/Udel-1 (Fig. 1G). The fact that in all three cases the







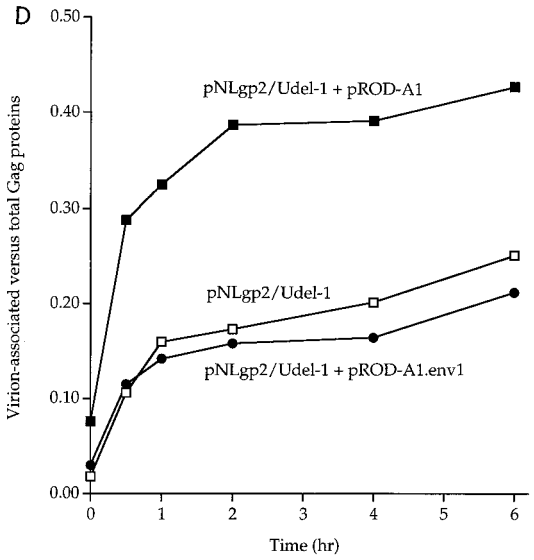


FIG. 4. HIV-2_{ROD} viral release is regulated by its envelope protein. (A to C) Kinetic analysis of protein synthesis and maturation and release of viral particles by pNLgp2/Udel-1 in the absence (A and C) or presence of gp140. HeLa cells were transfected with 20 μg of pNLgp2/Udel-1 plasmid DNA alone (A) or in the presence of 15 μg of pROD-A1 (B) or the envelope-deficient mutant pROD-A1.env1 (C). Pulse-chase analysis was done as described for Fig. 1. Viral proteins were recovered by immunoprecipitation of both the cell lysate (cell) and the pelleted virus (virus) fractions, separated on SDS–12% polyacrylamide gels, and visualized by fluorography. The positions of the HIV-1 gp160 and gp120 envelope glycoproteins and HIV-2 $p58^{gag}$, p26-27^{CA}, and $p16^{MA}$ are shown on the left. (D) Bands corresponding to the precursor and mature Gag proteins in panels A to C were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time. (E and F) Kinetics of viral particle release by an envelope mutant of the pROD10 isolate. HeLa cells were transfected with 20 μg of pROD10 (E) or the envelope-deficient pROD10.env1 mutant (F). Pulse-chase analysis, immunoprecipitation, and gel electrophoresis were performed as described for Fig. 1. The positions of the HIV-2 gp140 envelope glycoprotein and $p58^{gag}$, p26-27^{CA}, and $p16^{MA}$ are shown on the left. (G) Bands corresponding to the precursor and mature Gag proteins in panels E and F were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time. Particle release by *vif*, *vpr*, *vpx*, and *nef* mutants of pROD10 was assessed in identical pulse-chase and immunoprecipitation experiments (not shown) and quantified with an image analyzer. The ratio of virion-associated versus total Gag proteins, calculated as described for Fig. 1, was plotted as a function of time. (H) HeLa cells were transfected with 20 μg of pROD10.env1 in the presence of 10 μg of either pROD-A1, pCM5-Env (expressing only the ROD10 envelope glycoprotein), or the envelope-deficient mutant pROD-A1.env1 plasmid. Cells were pulse-labeled for 30 min with Trans^{35}S-Label and subjected to a 4-h chase. Cell- and virus-associated viral proteins were immunoprecipitated, separated on SDS-polyacrylamide gels, and visualized by fluorography (data not shown). Bands corresponding to the precursor and mature Gag proteins were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.

decreased efficiency of particle release could be accounted for by interference with the expression, maturation, or subcellular localization of the ROD10 envelope glycoprotein presents strong evidence that the ROD10 Env product, like Vpu, has the inherent capacity to enhance the release of progeny virions from virus-producing cells.

DISCUSSION

Although HIV-1 and HIV-2 are similar in genome organization, restriction site polymorphism as well as sequence analysis have revealed that HIV-2 is more closely related to certain

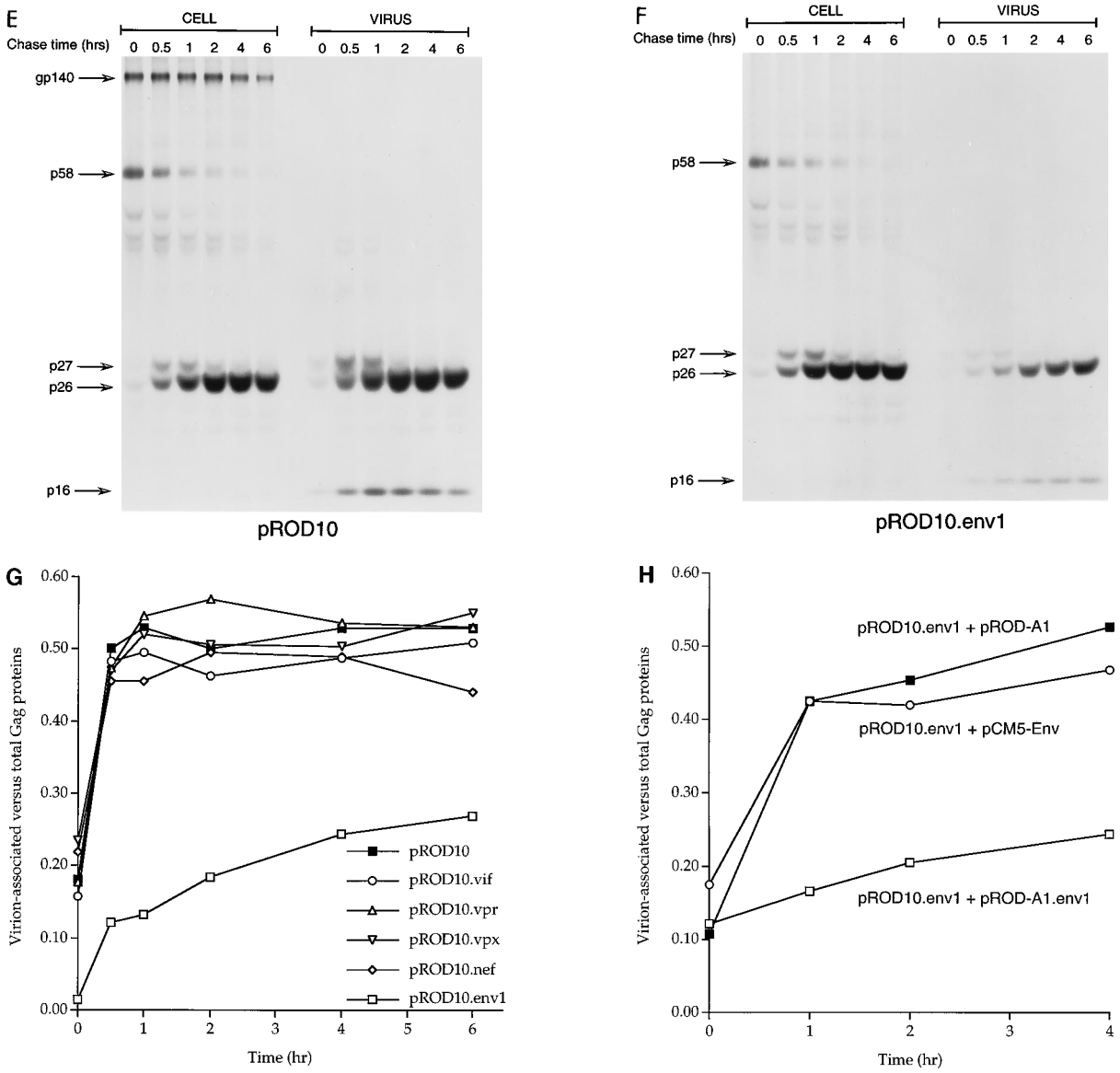


FIG. 4—*Continued.*

simian immunodeficiency virus strains than to HIV-1 (9). The main difference in the genome organizations of HIV-1 and HIV-2 is the presence of the *vpx* gene in HIV-2, which is absent in HIV-1, and the absence of the *vpu* gene in HIV-2, which is present in HIV-1. Even though it is tempting to speculate that the *vpx* gene in HIV-2 functionally replaces the HIV-1 *vpu* gene, the Vpx and Vpu are structurally unrelated, and data presented in this paper argue against a functional similarity between the two proteins. Aside from these structural differences, HIV-2 is known to be significantly less pathogenic than its HIV-1 counterpart (31). Even though HIV-2 infections are widespread, especially in West Africa (22, 38), most of the HIV-related clinical manifestations worldwide are due to infection with HIV-1 rather than HIV-2. The reasons for the different pathogenic potentials of HIV-1 and HIV-2 are unclear. However, it cannot be ruled out that the differential expression of *vpu* and *vpx* genes in HIV-1 or HIV-2 contributes to this phenomenon.

In our previous analyses of the function of the HIV-1 Vpu protein, we have routinely discussed the question of why only HIV-1 has evolved to encode a gene, *vpu*, that has two distinct functions, i.e., the rapid ER degradation of CD4 receptor (55, 56) and the enhancement of virus release from infected cells (49–51), which, at the same time, reduces HIV-induced cytopathic effects (25). With regard to Vpu-mediated CD4 degradation, we argued that the main purpose of this function might be to prevent the formation of stable intracellular complexes that block both CD4 and the HIV-1 Env protein in the ER and interfere with their cell surface expression (56). Since it is known that the affinity of HIV-2 Env for CD4 is between 10- and 200-fold lower than that of HIV-1 Env (2, 23, 34), we speculated that such a function of Vpu might simply not be required for efficient HIV-2 Env transport to the cell surface.

With regard to Vpu-mediated enhancement of virus secretion, no reasonable explanation for the lack of a Vpu-like factor in HIV-2 could be offered. The absence of a Vpu counterpart in HIV-2 seemed especially paradoxal in light of the fact that Vpu was shown to be able to enhance HIV-2 particle

release similar to that of HIV-1 (16). While the present study confirmed these findings, it also revealed that the effect of Vpu observed on the release of HIV-2 particles was influenced by the experimental design and was demonstrable only in the context of chimeric viruses. However, in the context of the wild-type ROD10 isolate of HIV-2, Vpu was unable to increase the rate of virus secretion, presumably because virus secretion in that case was already at a maximal level, with over 50% of newly synthesized viral proteins secreted within 6 h. Such a ratio was found to be similar to if not better than that for the prototypic Vpu^+ NL4-3 isolate. In fact, we observed that in the context of the chimeric NLgp2 and NLgp2/Udel-1 viruses, particle release mediated by HIV-2 Gag-Pol was slightly more efficient than the respective wild-type HIV-1 counterparts. Although this finding may indicate that HIV-2 Gag-Pol has a somewhat better inherent capacity than HIV-1 Gag-Pol to form viral particles, it cannot account for the high rate of particle release exhibited by pROD10.

The results of the experiments presented in this study add a new perspective to the question of why HIV-2 does not require a *vpu* gene by demonstrating that HIV-2 has indeed evolved a mechanism, although seemingly quite different from that of HIV-1, to regulate the release of its progeny virions. We presented evidence that the gp140 envelope protein of $HIV\text{-}2_{ROD}$, whether encoded by the pROD10 molecular clone or provided in *trans* by the pROD-A1 or pCM5-Env plasmid, can enhance particle release both in the context of HIV-1 chimeric viruses and the pROD10 molecular clone. The fact that Vpu can fully replace the gp140 envelope to enhance particle release by the NLgp2/Udel-1 virus demonstrates that the Vpu-like activity of the gp140 envelope protein does not simply reflect the requirement for HIV-2 Gag-Pol to interact with envelope proteins for efficient particle release. Although there is evidence in the literature that specific interactions between HIV-1 Gag and envelope proteins exist (11, 28, 35, 36, 58), it has also been shown that HIV-1 as well as HIV-2 particles can be formed in the absence of Env (17, 24, 26, 29, 33, 41, 53). Consistent with these results, we found that mutation of the HIV-1 envelope gene in both pNLgp2 and pNLgp2/Udel-1 had no effect on the efficiency of virus release by these viruses (6), indicating that the presence of envelope proteins is not a prerequisite for particle formation by HIV-2 Gag-Pol. It is thus likely that the mechanism by which the $HIV\text{-}2_{ROD}$ gp140 envelope protein enhances particle release relies on a new biological activity of this protein. From our work on Vpu, we know that the transmembrane (TM) domain of Vpu plays a crucial function in this process (44), and we have recent evidence that this domain of Vpu has the propensity to form ion channels in planar lipid bilayers (43). In addition, the ability of the Vpu TM domain to form ion-conductive membrane pores is directly correlated with its ability to enhance virus release from the cell surface (43). It will be interesting to see whether by analogy to Vpu, the virus release function of the HIV-2 Env protein can be similarly correlated with a potential ion channel activity of its TM domain. In this regard, it may be relevant that gp140 is, like Vpu, an integral membrane protein that forms homo-oligomeric structures (30, 39, 40). gp140 and Vpu are also similarly regulated, since in HIV-1, Vpu and Env are translated from the same bicistronic mRNA (48). In addition, both proteins are sensitive to BFA (reference 47 and this report), indicating that they may act in similar subcellular compartments. Alternatively, BFA may inhibit posttranslational modifications of the ROD10 Env, such as glycosylation or proteolytic cleavage, that may be necessary for activation of its activity on particle release. Finally, we have evidence that the ROD10 Env protein is capable of substituting for Vpu to regulate the release of HIV-1 particles (6). Mutagenesis studies of the $HIV\text{-}2_{ROD}$ *env* gene are under way and should lead to a more precise definition of the molecular mechanism involved in gp140-mediated enhancement of particle release.

It will be further interesting to see whether the ability to regulate virus release is a function that has evolved in HIV-2 Env in place of Vpu or whether it reflects a function that has been lost in HIV-1 because of Vpu. Even though published evidence suggests that HIV-1 Env does not affect virus particle release (14, 57), we are currently investigating whether HIV-1 Env has perhaps a residual activity on HIV-1 virus release which might indicate that such a capacity of HIV-1 Env did originally exist.

We do not know whether the Vpu-like activity of the HIV-2 envelope protein is a specific characteristic of the ROD10 isolate or whether it represents a generalized mechanism used by all HIV-2 strains. However, previous studies have shown that the efficiency of particle release directed from a full-length molecular clone of SIV (pFLB10) was two to three times higher than that of the HIV-1 Vpu^+ HXBc2 isolate (51). Moreover, particle release by this virus could not be enhanced by Vpu provided in *trans* (51). These results are reminiscent to our observations with the HIV-2 molecular clone pROD10 and further indicate that viruses that lack a *vpu* gene may nevertheless express Vpu-like activities to regulate viral particle release.

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